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Use of quantitative real-time PCR to determine the local inflammatory response in the intestinal mucosa and *muscularis* of horses undergoing small intestinal resection

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Abstract

Background: Studies in rodents and humans have demonstrated that intestinal manipulation or surgical trauma initiates an inflammatory response in the intestine which results in leucocyte recruitment to the *muscularis externa* causing smooth muscle dysfunction.

Objectives: To examine the intestinal inflammatory response in horses undergoing colic surgery by measuring relative differential gene expression in intestinal tissues harvested from surgical colic cases and control horses.

Study design: Prospective case-control study.

Methods: Mucosa and *muscularis externa* were harvested from healthy margins of resected small intestine from horses undergoing colic surgery (n = 12) and from intestine derived from control horses euthanised for reasons unrelated to the gastrointestinal tract (n = 6). Tissue was analysed for genes encoding proteins involved in the inflammatory response: *interleukin (IL) 6* and *IL1β*, *C-C motif chemokine ligand 2 (CCL2)*, *tumour necrosis factor (TNF)*, *prostaglandin-endoperoxide synthase 2 (PTGS2)* and *indoleamine 2,3-dioxygenase (IDO1)*. Relative expression of these genes was compared between the two groups. Further analysis was applied to the colic cases to determine whether the magnitude of relative gene expression was associated with the subsequent development of post-operative reflux (POR).

Results: Samples obtained from colic cases had increased relative expression of *IL1β*, *IL6*, *CCL2* and *TNF* in the mucosa and *muscularis externa* when compared with the control group. There was no difference in relative gene expression between proximal and distal resection margins and no association between duration of colic, age, resection length, short-term survival and the presence of pre-operative reflux and the relative expression of the genes of interest. Horses that developed POR had significantly greater relative gene expression of *TNF* in the mucosa compared with horses that did not develop POR.

Main limitations: Small sample size per group and variation within the colic cases.

Conclusions: These preliminary data support an upregulation of inflammatory genes in the intestine of horses undergoing colic surgery.

Neil P. H. Hudson, David A. Hume and R. Scott Pirie are joint senior co-authors.

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KEYWORDS

colic surgery, gastrointestinal, gene expression, horse, inflammation, macrophage

1 | INTRODUCTION

Studies on the margins of surgically resected equine intestine from horses undergoing colic surgery identified a generalised stress response in the smooth muscle and myenteric plexus, characterised by an increase in apoptotic smooth muscle cells and apoptotic neurons and glial cells.¹ Ischaemia and manipulation of the equine jejunum also induces a post-operative neutrophilic infiltration in all tissue layers of the jejunum and eosinophilic infiltration of the jejunal submucosa.^{2,3} In an equine model where the jejunum was distended and then decompressed, there was also an increase in the number of neutrophils in the mucosa and the serosa.⁴ This inflammatory infiltrate is likely a downstream consequence of resident intestinal inflammatory cell activation in the *muscularis externa* that has been described in rodents and humans.⁵⁻⁷

Intestinal manipulation triggers the activation of *muscularis externa* macrophages (MM) by pathogen-associated molecular patterns such as lipopolysaccharide.⁸ This results in cytokine and chemokine release followed by the infiltration of leucocytes, predominantly neutrophils, monocytes and mast cells to the *muscularis externa*. These infiltrating leucocytes secrete products, such as nitric oxide and prostaglandins which can impair smooth muscle function.^{9,10} Genes associated with inflammation of the *muscularis externa* induced by manipulation or surgery in rodent models and human studies include the pro-inflammatory cytokines *interleukin (IL) 6*, *IL1 β* , *tumour necrosis factor (TNF)*, *nitric oxide synthase 2 (NOS2)*, the chemokine *C-C motif chemokine ligand 2 (CCL2)* and the immunomodulatory enzyme mediator *prostaglandin-endoperoxide synthase 2 (PTGS2)*.^{7,11} This inflammatory response within the *muscularis externa* is strongly implicated as a significant causative factor in the pathogenesis of post-operative ileus (POI).⁵ Despite the high prevalence of POI in horses following colic surgery, there are no equine studies to date evaluating the inflammatory response genes known to be associated with the development of POI in rodent and human studies. We hypothesised that an increase in inflammatory gene expression of *IL6*, *IL1 β* , *CCL2*, *TNF*, *PTGS2* and *indoleamine 2,3-dioxygenase (IDO1)* will be present in the resected margins of horses undergoing small intestinal resection. Furthermore, we hypothesised that inflammatory gene expression will be greater in horses that subsequently develop post-operative reflux (POR) (a characteristic feature of POI) following abdominal surgery, compared with those that do not.

2 | MATERIALS AND METHODS

2.1 | Study design

This was a prospective case-control two-centre study between October 2014 and February 2017. Control horses were presented for elective euthanasia for a variety of reasons unrelated to diseases

and disorders of the intestinal tract; these included poor temperament, chronic orthopaedic conditions, recurrent laminitis and suspected dental disease. Additionally, financial constraints of the owners contributed towards the decision for elective euthanasia in some of these cases. Control horses were excluded if any gross gastrointestinal lesions were identified at post-mortem examination. Horses were euthanased with secobarbital sodium 400 mg/mL and cinchocaine hydrochloride 25 mg/mL (Somulose; Dechra Veterinary Products) and samples were obtained approximately 30 minutes following euthanasia. The colic case group comprised horses over 1 year of age undergoing exploratory celiotomy for colic unresponsive to medical management that required resection of a segment of the small intestine. Case inclusion was also dependent on the feasibility of appropriate sample collection and processing by the surgeon on duty. One experienced surgeon from each centre performed the exploratory celiotomies, intestinal resections and sample collection. All colic cases received the same pre- and post-operative care (Data S1).

Signalment was recorded in both control horses and colic cases. For each colic case, site of lesion and resection, duration of colic prior to surgery, presence of pre-operative reflux, resection length, short-term survival (defined as surviving to hospital discharge) and the presence of POR were recorded. Pre-operative reflux was defined as the presence of >2 L of reflux obtained on passing a nasogastric tube prior to surgery and POR was defined as the presence of >2 L of reflux on more than 2 intubations in the post-operative period. Both owner consent and ethical approval were obtained for the collection of tissues; ethical approval was provided by the Royal (Dick) School of Veterinary Studies Veterinary Ethical Review Committee.

2.2 | Sample collection

Control samples were obtained from an antimesenteric location in the region of the mid jejunum. For the colic cases, the resected intestine was deemed by the surgeon to have healthy grossly viable resection margins. Samples were obtained from both proximal and distal resection margins of either jejunum or ileum. All proximal resection margins were obtained from the jejunum and distal resection margins were either obtained from the jejunum ($n = 9$) or ileum ($n = 3$).

For both the control and colic samples, full thickness sections of intestine were excised and rinsed in cold phosphate-buffered saline (PBS) (Dulbecco's Phosphate Buffered Saline; Sigma-Aldrich). The mucosa and *muscularis externa* were separated, cut into segments and stored in RNALater (Sigma-Aldrich) at 4°C for up to 30 days, prior to long-term storage at -80°C. For the control horses, additional sections were excised adjacent to the sample collection site, fixed in formalin and embedded in paraffin prior

to staining with haematoxylin and eosin to confirm the absence of any histopathological change indicative of pre-existing underlying pathology.

2.3 | RNA extraction and cDNA synthesis

Tissue sections were thawed, then homogenised in the presence of β -mercaptoethanol and transferred to a gDNA Eliminator column (Qiagen) before extraction of total RNA using an RNeasy Plus Mini kit (Qiagen). RNA was quantified and purity was assessed using a Nanodrop spectrophotometer (ThermoFisher Scientific) and integrity was measured using the TapeStation System (Agilent Technologies). An RNA integrity number (RIN[®]) greater than 7 was considered sufficient for downstream analysis¹² and cDNA was synthesised from 1 μ g of total RNA using the SuperScript III First-Strand synthesis system (Invitrogen).

It was not possible to extract RNA of adequate quality from all the colic cases and control samples. A total of 9 *muscularis externa* samples (5 proximal and 4 distal) and 16 mucosal samples (8 proximal and 8 distal) from colic cases and 4 *muscularis externa* and 6 mucosal samples from control horses were used for RT-qPCR analysis. For colic cases, samples from the proximal and distal resection margins were analysed. After preliminary analysis comparing relative gene expression between proximal and distal resection margins with control tissues, the proximal mucosa (n = 8) and distal mucosa (n = 8) samples were combined as were the proximal and distal *muscularis externa* sections (Figure 1). Proximal and

distal margins obtained from the same case were not used. Where a proximal and distal sample existed for a colic case, the proximal section was selected as all proximal resection margins were from the jejunum and therefore better matched the control samples (Table S1).

2.4 | Relative gene expression analysis

The relative expression of *IDO1*, *IL6*, *IL1 β* , *TNF*, *CCL2*, *NOS2* and *PTGS2* was analysed using real-time quantitative polymerase chain reaction (RT-qPCR).

For RT-qPCR, cDNA was amplified with Power SYBR Green PCR Master Mix using the 7500 fast Real Time PCR system (ThermoFisher Scientific). Primer efficiency was validated with a standard curve of five serial dilution points (three for *NOS2*) with efficiency ranging between 92.68% and 100.74%. Amplification reactions were performed in triplicate and the melting curve analysed to check the annealing temperature and to ensure that there was no primer dimer formation. Efficiency and reactions were analysed using 7500 Software v2.3 (ThermoFisher Scientific). Details of primer sequences and design are included in Table S2.

The expression of target gene mRNA relative to the housekeeping gene, *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*), was calculated for each sample using the $2^{-(\Delta\text{threshold cycle (CT)})}$ ($2^{-\Delta\Delta\text{CT}}$) method.¹³ Relative gene expression was assessed in the mucosa and *muscularis externa* of samples from colic cases and control horses.

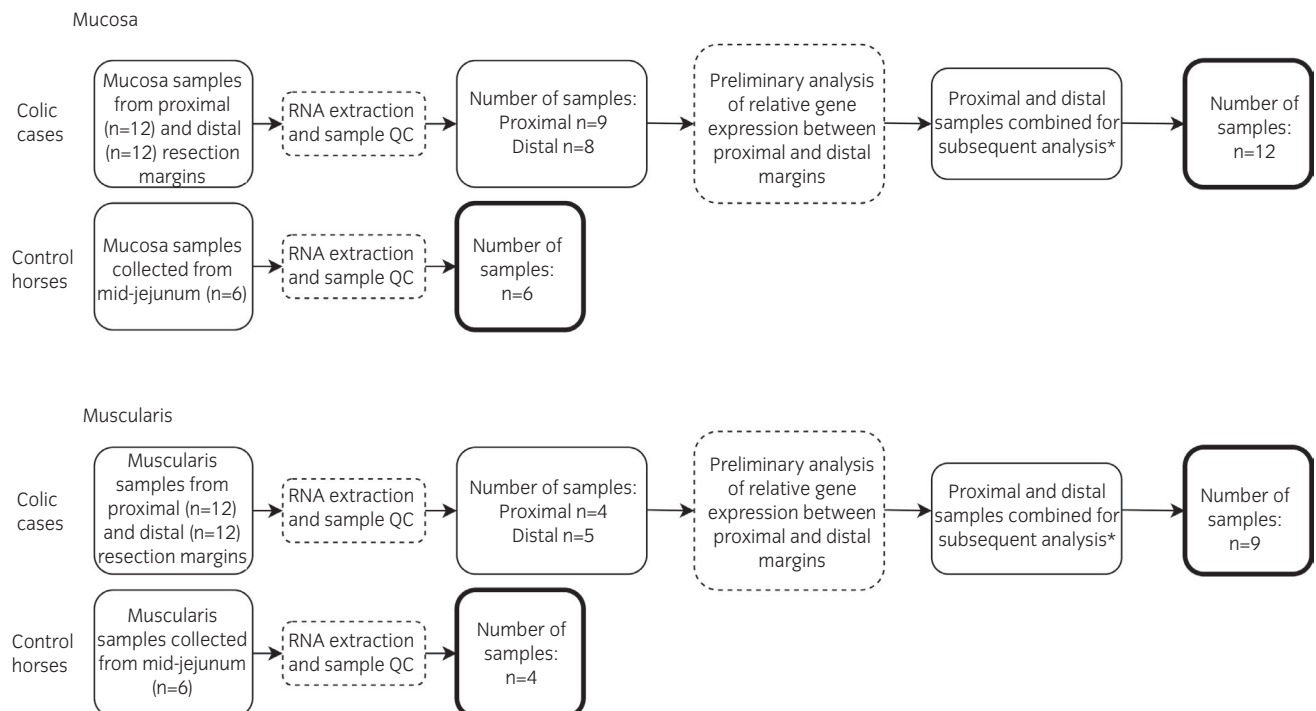


FIGURE 1 Flow chart describing process of sample selection following quality control and preliminary analysis. *For each case, either a proximal or distal sample was selected

2.5 | Data analysis

Where appropriate, data are reported as median and interquartile range. All statistical analyses were performed using GraphPad Prism 8.4.3 (GraphPad Software; GSL Biotech LLC). A non-parametric Mann-Whitney U test was applied to determine significant differences in relative gene expression between proximal and distal margins of the mucosa and *muscularis externa*; the mucosa samples obtained from the colic cases and the control horses; the *muscularis externa* samples obtained from the colic cases and the control horses; horses that did and did not survive to discharge; and horses with and without pre- and post-operative reflux. Linear regression analysis was used to analyse the relationship of age, duration of colic and resection length with relative gene expression. Normality was measured using D'Agostino's K^2 test. Significance was assumed at $P < .05$.

3 | RESULTS

The colic cases comprised 12 horses (median age 19.5 years, range: 9-27 years) of various breeds and included 6 males (5 geldings, 1 stallion) and 6 mares. All cases had compromised small intestine, the gross appearance of which necessitated surgical resection. Surgical lesions included strangulation of the jejunum ($n = 7$) and ileum ($n = 1$) by a pedunculated lipoma, epiploic foramen entrapment ($n = 2$), eosinophilic enteritis ($n = 1$) and strangulation of the jejunum by the gastrosplenic ligament ($n = 1$). Nine colic cases survived to discharge. The controls comprised six horses (median age 18 years; range: 15-23 years) of various breeds and consisted of three geldings and three mares. Horse details are summarised in Table S3.

In relation to the analysed mucosa samples, median age of colic cases was 20.5 years (range: 10-27 years) with a median duration of colic of 6.5 hours (range: 2-12 hours) and resection length of 1.8 metres (range: 0.5-1.5 metres). Four colic cases had pre-operative reflux and three developed POR. With respect to the analysed *muscularis externa* samples, median age was 12 years (range 9-27 years) with a median duration of colic of 4 hours (range: 2-5 hours) and median resection length of 0.5 metres (range: 0.5-1.8 metres). Four colic cases had pre-operative reflux and three horses developed POR.

3.1 | Relative expression of target genes in the mucosa and muscularis externa of colic cases and controls

No significant differences in relative gene expression of target genes were observed between proximal and distal resection margins for both the mucosa and *muscularis externa* prior to the samples being combined (Figures S1 and S2). *NOS2* was removed from the set of target genes as no mRNA was detectable in any of the mucosa and *muscularis externa* samples.

Relative expression of the target genes was evaluated in the mucosa and *muscularis externa* of colic cases and compared with the mucosa and *muscularis externa* of control horses (Figure 2). In the mucosa, the level of relative expression of *IL1 β* , *IL6*, *PTGS2*, *TNF* and *CCL2* was greater ($P < .05$) in colic cases compared with control horses. In the *muscularis externa*, the level of relative expression of *IL1 β* , *IL6*, *TNF* and *CCL2* was greater in colic cases compared with control horses ($P < .05$). There was no difference in the relative expression of *IDO1* in colic cases, compared with control horses, with the exception of one colic case (Case C), which had a 300-fold greater relative expression of *IDO1* in the mucosa compared with the other colic cases.

The outlying data points are from four colic cases: A, C, D and J. Case A had higher relative gene expression for *IL1B*, *IL6*, *PTGS2* and *CCL2* in the mucosa, Case C had higher relative gene expression for *PTGS2* and *CCL2* in the mucosa, Case D had higher relative gene expression of *IL1B*, *TNF* and *CCL2* in the *muscularis externa* and Case J had higher relative gene expression of *IL1B*, *IL6*, *PTGS2*, *TNF* and *CCL2* in the mucosa and *TNF* in the *muscularis externa*. Case F and Case K also had one outlying data point each.

3.2 | Relative expression patterns of target genes across individual horses

A comparison of relative gene expression levels in each individual colic case, across all analysed target genes, showed that those with the highest expression in one target gene also exhibited a relatively greater expression of the other target genes in both the mucosa and *muscularis externa* (Figure 3).

3.3 | Analysis of relative expression in colic cases with relation to age, duration of colic, resection length, short-term survival and the presence of pre- and post-operative reflux

There were no significant associations in either the mucosa or *muscularis externa* between inflammatory gene relative expression and the following factors: presence of pre-operative reflux; length of resected intestine; survival to discharge and duration of colic.

In the mucosa, relative expression of all genes, except for *TNF*, reduced with age. There was a significant decrease in the relative expression of *PTGS2* and *CCL2* with increasing age ($P < .05$) (Figure S3). In the *muscularis externa*, all genes, with the exception of *IDO1*, showed a trend of increased relative expression with increasing age; however, this apparent association was not statistically significant (Figure S4). Mucosal samples from colic cases that developed POR demonstrated a significantly greater relative expression of *TNF* when compared with mucosal samples from colic cases that did not develop POR (Figure 4). There was no statistically significant difference in relative gene expression in the *muscularis externa* of

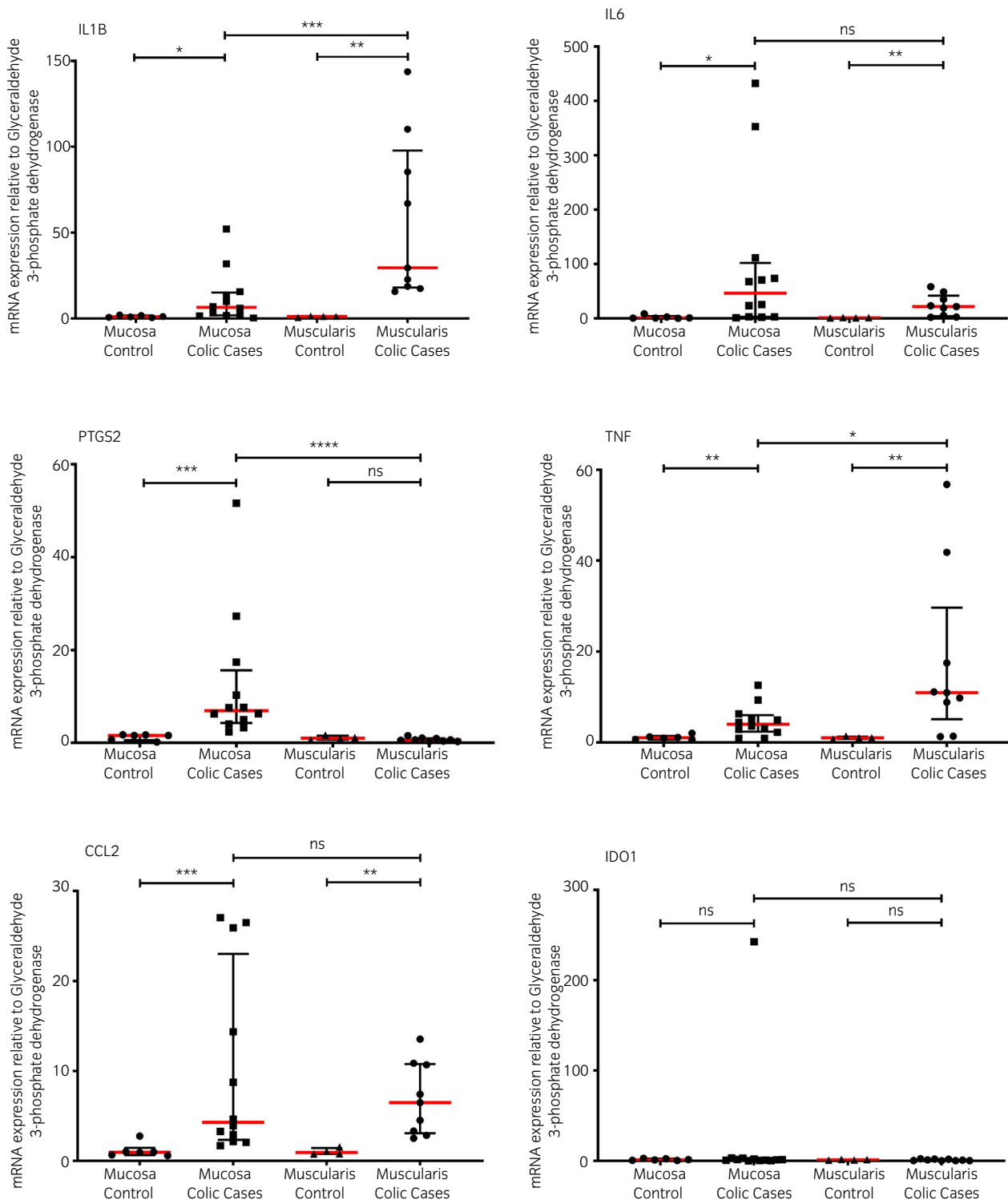


FIGURE 2 Relative gene expression of *IL1B*, *IL6*, *PTGS2*, *TNF*, *CCL2* and *IDO1* in the mucosa and *muscularis externa* of horses undergoing colic surgery. Scatter plots showing relative mRNA expression of target genes relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the distal mucosa ($n = 12$) and combined proximal and distal *muscularis externa* ($n = 9$) samples compared with controls (mucosa $n = 6$, *muscularis externa* $n = 4$). Significance of relative gene expression levels between control and surgical samples using a Mann-Whitney U test. ns = not significant, * $P < .05$, ** $P < .01$, *** $P < .001$, **** $P < .0001$

colic cases that developed POR when compared with colic cases that did not develop POR (Figure 5); however, those that developed POR showed a greater median relative expression of *IL1 β* , *IL6*, *PTGS2*, *TNF* and *CCL2*.

4 | DISCUSSION

The principal aim of this study was to examine the intestinal inflammatory response in both the mucosa and *muscularis externa*

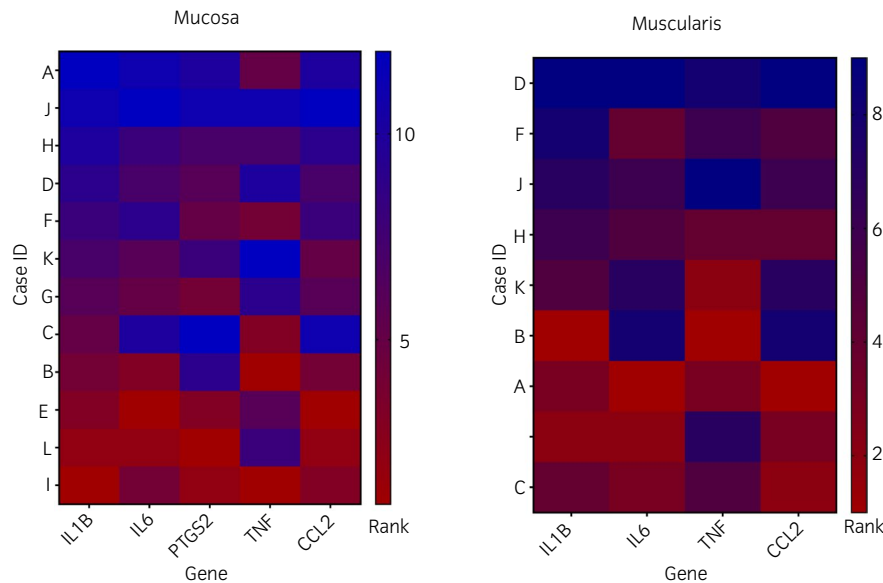


FIGURE 3 Heatmap of ranked relative gene expression in the mucosa and *muscularis externa* of horses undergoing colic surgery. The level of relative gene expression was ranked for all genes that were significantly increased in colic cases in the mucosa (*IL1B*, *IL6*, *PTGS2*, *TNF* and *CCL2*) and *muscularis externa* (*IL1B*, *IL6*, *TNF* and *CCL2*). Number 1 was assigned as the lowest rank, representing the lowest difference in relative gene expression per gene. In the mucosa, Cases A and J had the largest difference in relative gene expression for all the genes, except for *TNF* in case A. Cases I and L both had the smallest difference in relative expression across all the genes, with the exception of *TNF* in Case L. In the *muscularis externa*, Case D has the highest ranking due to having the largest gene expression increase in all genes, followed by F and J

of horses undergoing abdominal surgery that necessitated intestinal resection. By measuring differential gene expression of a set of target genes (*IDO1*, *IL6*, *IL1 β* , *TNF*, *CCL2*, *NOS2* and *PTGS2*), we demonstrated the presence of intestinal inflammation in horses undergoing colic surgery. This gene list selection was based on previous reports of their upregulation in the *muscularis externa* of murine POI models¹⁴ and in humans undergoing laparotomy.⁷ In rodent intestinal manipulation-induced POI models, activation of MM results in cytokine and chemokine release, followed by infiltration of leucocytes into the *muscularis externa*. These infiltrating leucocytes inhibit smooth muscle function via the secretion of leucocytic products, such as nitric oxide (NO) and prostaglandins. This macrophage activation, which occurs within hours of surgery, has also been demonstrated in human patients.⁷ As well as playing a key role in the initiation of inflammation, macrophages also have a protective function and are pivotal to the tissue repair process.¹⁴ At the level of the intestine, this protective function was recently demonstrated in a rodent model of infection-induced inflammation, in which *muscularis externa* macrophages protected the enteric neurons against post-infection neuronal death.¹⁵

IL6, *IL1 β* , *CCL2* and *TNF* were all upregulated in both the mucosa and *muscularis externa* in colic cases, whereas *PTGS2* was upregulated in the mucosa only. These findings demonstrate the presence of active inflammation within intestinal tissue visually assessed as non-devitalised, a finding consistent with data derived from the *muscularis externa* of humans undergoing laparotomy.⁷ In addition, we also identified an inflammatory cytokine and mediator response within the mucosa, a finding which contrasts with the results

derived from a study of people undergoing laparotomy which failed to show upregulation of *IL6*, *PTGS2* and *NOS2* mRNA within the mucosa.⁷ This disparity could, however, be attributed to the difference between the two sample populations. The study by Kalff et al⁷ comprised subjects undergoing elective abdominal surgery for colectomy, stoma repair or resection of intestine due to adhesions, neoplasia or strictures; in comparison, our cases underwent emergency abdominal surgery that necessitated resection of devitalised small intestine. Our failure to identify any significant differences in relative gene expression (mucosa and *muscularis externa*) between the proximal and distal resection margins in the colic group contrasts with the findings of Gerard et al, who reported a significantly greater number of infiltrating neutrophils within the jejunal mucosa of proximal resection margins compared to both the distal margin and control tissue.¹⁶ It is possible that this increased neutrophil infiltration in the proximal resection margins may, at least in part, be attributable to intestinal dilation, consistent with the findings of several studies describing the injurious effect of experimental luminal dilation, resulting in mucosal injury, smooth muscle oedema and neutrophilic infiltration.^{4,16–18} As smooth muscle oedema and leucocyte infiltration can both inhibit intestinal motility,^{6,14,19} it is understandable how such events can contribute towards the development of POI. The fact that our data failed to reveal any difference in the relative expression of inflammatory genes between the proximal and distal resection margins suggests that intestinal dilation had little or no effect on the intestinal inflammatory response in the horses included in our study. The inability to detect any *NOS2* mRNA in any of the tissue sections (control and colic

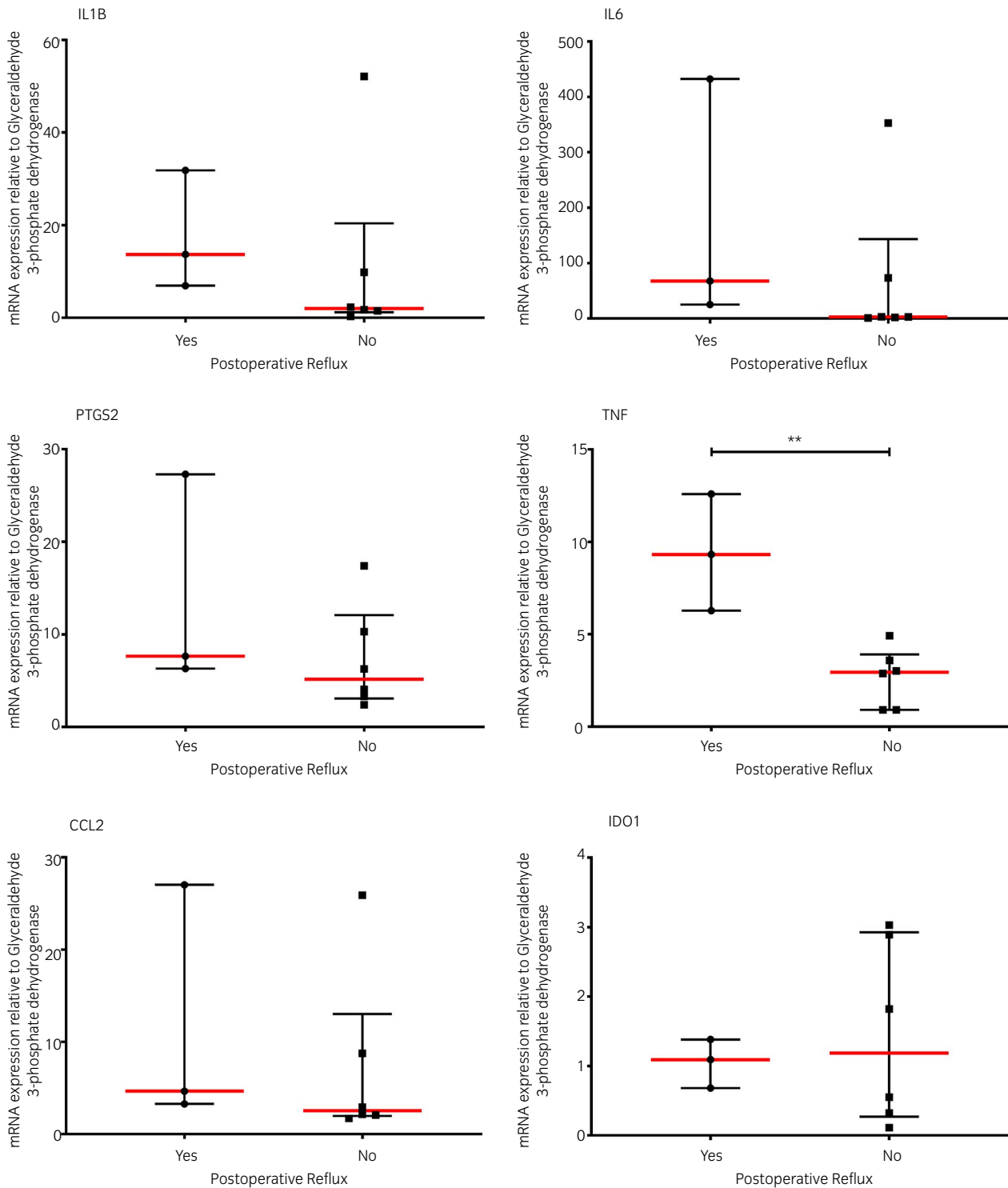


FIGURE 4 Relative gene expression of *IL1B*, *IL6*, *PTGS2*, *TNF*, *CCL2* and *IDO1* in the mucosa of horses with and without post-operative reflux. Scatter plots showing mRNA expression of target genes relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the mucosa of colic cases ($n = 9$) and horses that developed post-operative reflux ($n = 3$). Significance of relative gene expression levels in horses with pre-operative reflux was assessed using a Mann-Whitney U Test. * $P < .05$

cases) highlights a key difference in the innate immune response between the horse and rodents. Rodents are able to produce nitric oxide (NO), through the metabolism of arginine, via the inducible enzyme NO synthase 2, encoded by the *Nos2* gene.²⁰ Intestinal manipulation in rodents causes an increase in *Nos2* mRNA in both the

mucosa and *muscularis externa* and an associated increase in NO production which mediates smooth muscle dysfunction resulting in POI.²¹ The lack of *NOS2* mRNA in this study supports previous findings where equine bone-marrow-derived macrophages failed to produce NO in response to stimulation with the endotoxin,

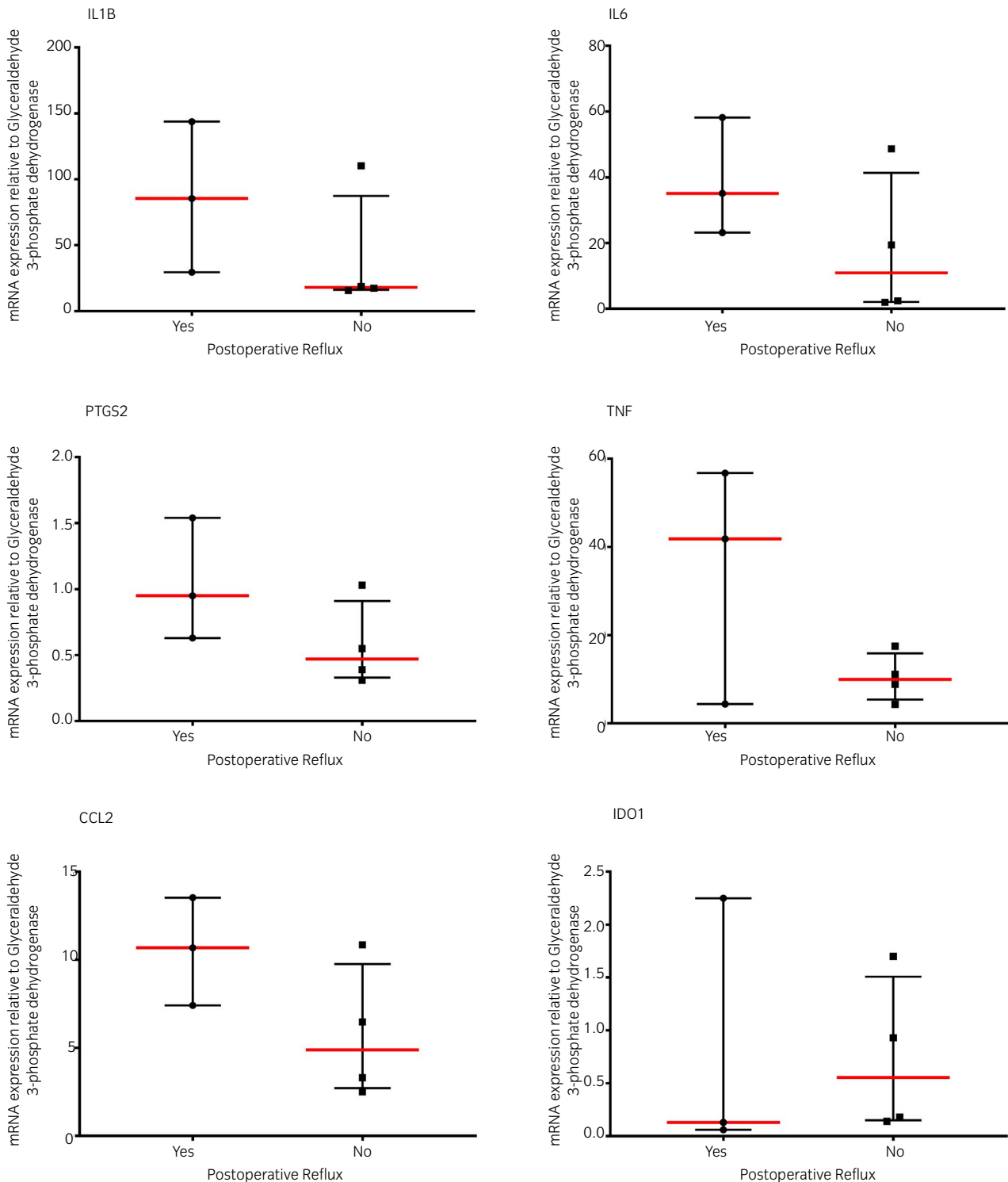


FIGURE 5 Relative gene expression of *IL1B*, *IL6*, *PTGS2*, *TNF*, *CCL2* and *IDO1* in the muscularis externa of horses with and without post-operative reflux. Scatter plots showing mRNA expression of target genes relative to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in muscularis externa of colic cases ($n = 7$) and horses that developed post-operative reflux ($n = 3$). Significance of relative gene expression levels in horses with pre-operative reflux was assessed using a Mann-Whitney U Test

lipopolysaccharide, and did not upregulate any genes involved in arginine metabolism.²² These data, and the data from this study suggest that NO production from macrophages does not contribute to the pathophysiology of equine POI.

The cytokines *IL1 β* and *TNF α* reduce intestinal motility via a reduction in smooth muscle function, either by direct action on the smooth muscle cell or an indirect suppression of neurotransmitter release.^{23,24} *IL6* also suppresses motility, although the exact

mechanisms that underpin this effect are currently unknown.²⁵ Although *CCL2* does not directly affect neurotransmission or smooth muscle function, upregulation of this cytokine may exert an indirect effect on motility via its recruitment of monocytes and leucocytes. When inflamed, both the mucosa²⁶ and *muscularis externa*²⁷ show an increase in *CCL2* expression. Infiltrating neutrophils and monocytes can impair smooth muscle contraction via the secretion of NO and prostaglandins.¹⁰ In rodents, *PTGS2* inhibits smooth muscle function,²⁸ an effect supported by the increased contractility of human jejunal circular muscle following inhibition of the *PTGS2* pathway.⁷ These findings somewhat contradict the failure to identify any increase in the relative expression of *PTGS2* in the *muscularis externa* of horses undergoing abdominal surgery, despite a significant increase in the mucosa. This may be attributable, at least in part, to the timing of sample collection relative to gene expression which, in turn, will relate to the type and magnitude of cell infiltrate at the time of the resection; it is possible that cells in the mucosa are activated sooner than those within the *muscularis externa*.

In contrast to other inflammatory markers (with the exception of one horse), there was no detectable difference in the relative expression of *IDO1* between the colic cases and control horses. Although *IDO1* induction can be mediated through both IFN- γ -dependent and -independent pathways, IFN- γ is the most potent inducer of both *IDO* gene expression and enzyme activity.²⁹ Furthermore, the LPS-mediated induction of *IDO* can be further potentiated via inflammatory cytokines such as *TNF*, *IL6* and *IL1 β* .^{30,31} Therefore, the lack of *IDO1* induction in the current study may have been attributable to a failure of any surgically induced activation of the IFN- γ pathway; alternatively, there may have been insufficient time for the induction of *IDO* via IFN- γ -independent pathways (LPS/*TNF*) prior to sample collection.

Several factors such as age and the presence of pre-operative nasogastric reflux have been associated with an increased incidence of POI in horses.³² In rodent models, a greater inflammatory response is associated with more "severe" POI.³³ Analysis was performed to determine if any association with pre-, intraoperative and post-operative factors was present. In the intestinal mucosa, there was a trend towards a reduction in the relative expression of all target genes, with the exception of *TNF* with age. The reduction in the relative expression of *PTGS2* and *CCL2* was statistically significant. In contrast, the relative expression of all target genes in the *muscularis externa* increased with age although 6 out of the 12 colic cases were diagnosed with pedunculated lipomas, the incidence of which is significantly greater in older horses.³⁴ In contrast, duration of colic was associated with a non-significant decrease in relative gene expression across all genes in both the mucosa and *muscularis externa*. Despite the observed trends and statistical results, the small sample size and large variation at both the individual horse and disease level preclude any definitive conclusions being made. Further studies incorporating a larger sample size and potentially a more uniform disease definition are clearly warranted.

The outlying data points were predominantly from four cases. There was no evidence of any differences in disease process or signalment to account for these phenomena. The study of larger group

sizes may have revealed specific factors associated with a greater inflammatory response, thus accounting for the response observed in the outlying individuals. It is also possible that host genetics may play a role in the severity of inflammation and, like that seen in humans, some individuals may be genetically predisposed of developing a more severe inflammatory response.³⁵

The main limitations of the current study were the small group sizes. The inclusion of larger group sizes would be required to definitively explore the validity of certain association trends identified by the current study. Based on our preliminary data, power calculations revealed that a minimum group size of 25-60 horses (depending on gene of interest) would be required to detect a significant correlation between relative gene expression and horses that do develop POR. Although the use of combined proximal and distal resected margin samples could also be considered as a limitation, preliminary analysis failed to reveal any statistically significant differences in relative gene expression between these different locations. In the colic case group, there was also variation in the length of resected intestine, the underlying disease process, the disease duration prior to sample collection and the age of the horses. Although these sources of variation will inevitably impact upon the interpretation of the results, they are difficult to control within the constraints of the field study nature of the study design. Lastly, some of the distal resection margins were obtained from the ileum (3 out of 12 cases), which may have different resident inflammatory cell numbers compared with the control jejunal samples. While we have previously shown a difference between distal jejunum and ileum with respect to macrophage numbers in the submucosa, this difference was not evident in other layers including the *muscularis externa*.³⁶ That said, quantification of other resident immune cells in the equine intestine has not been performed and remains a significant knowledge gap. A further limitation of the study relates to the inherently restricted inferences which can be derived solely from consideration of gene expression data; this may not fully mirror the protein transcription profile at the tissue level. Expansion of this work to include proteomic profiling and pathway analyses would be warranted to more fully elucidate the factors involved in the initiation and propagation of the inflammatory response.

While correlation of relative gene expression with several perioperative factors (e.g. development of POR) would have added further clinical relevance to the study, this was considered an adjunct to the primary objective of the study; namely, to extend findings from rodent and human studies to samples obtained from horses undergoing abdominal surgery. In this respect, our data confirmed the presence of an inflammatory response, characterised by differential inflammatory gene expression, in the intestine of horses undergoing colic surgery. As such, these findings justify continued research in this area and provide a methodological platform for such. The inclusion of a larger cohort of horses would greatly facilitate any future efforts to further assess whether associations exist between the magnitude of the inflammatory response and certain clinically relevant factors such as age, resection length, duration of colic, short- and long-term survival and the

development of POR. Furthermore, further work to determine the roles of different cell types such as neutrophils and macrophages in intestinal inflammation is also warranted.

5 | CONCLUSIONS

These data demonstrate an inflammatory response within the intestine of horses undergoing colic surgery during which a small intestinal resection was performed. The upregulated genes encode proteins with the capability of inhibiting smooth muscle contractility and disrupting normal intestinal motility, ultimately resulting in functional POI. Additionally, the absence of *NOS2* upregulation highlights inter-species variation in this inflammatory pathway, thus emphasising the importance of considering the target species when developing potential therapeutic targets. This study provides a foundation for future work to improve our understanding of the inflammatory response in horses undergoing colic surgery.

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CONFLICT OF INTERESTS

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

Z. Lisowski, R.S. Pirie, N. Hudson and D. Hume conceptualised, designed and interpreted the data in this study. Z. Lisowski, L. Lefevre, E. Clark and T. Mair contributed to the acquisition of the data. Z. Lisowski and L. Lefevre analysed and interpreted the data. Z. Lisowski prepared the manuscript and all authors contributed to, revised and approved the final manuscript.

ETHICAL ANIMAL RESEARCH

Tissue collection was approved by the Royal (Dick) School of Veterinary Studies' (R(D)SVS) Veterinary Ethical Review Committee.

INFORMED CONSENT

Owners gave consent for their animals' inclusion in the study.

DATA ACCESSIBILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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